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axl, a Transforming Gene Isolated from Primary Human Myeloid Leukemia Cells, Encodes a Novel Receptor Tyrosine Kinase

JOHN P. O'BRYAN,[1] ROY A. FRYE,[2] PATRICIA C. COGSWELL,[2] ANDREAS NEUBAUER,[2]†
BARRY KITCH,[2] CAROL PROKOP,[2] RAFAEL ESPINOSA III,[3] MICHELLE M. LE BEAU,[3]
H. SHELTON EARP,[2] AND EDISON T. LIU[1,2]*

Curriculum in Genetics[1] and Department of Medicine,[2] Lineberger Cancer Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, and Section of Hematology/Oncology, Department of Medicine, The University of Chicago, Chicago, Illinois 60637[3]

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Using a sensitive transfection-tumorigenicity assay, we have isolated a novel transforming gene from the DNA of two patients with chronic myelogenous leukemia. Sequence analysis indicates that the product of this gene, *axl*, is a receptor tyrosine kinase. Overexpression of *axl* cDNA in NIH 3T3 cells induces neoplastic transformation with the concomitant appearance of a 140-kDa *axl* tyrosine-phosphorylated protein. Expression of *axl* cDNA in the baculovirus system results in the expression of the appropriate recombinant protein that is recognized by antiphosphotyrosine antibodies, confirming that the *axl* protein is a tyrosine kinase. The juxtaposition of fibronectin type III and immunoglobulinlike repeats in the extracellular domain, as well as distinct amino acid sequences in the kinase domain, indicate that the *axl* protein represents a novel subclass of receptor tyrosine kinases.

Receptor tyrosine kinases represent a class of proteins that transduce signals from the extracellular milieu into the cytoplasm by binding peptide growth factors. These growth factors and their cognate receptors are involved in regulating cellular growth and differentiation. Alteration of either growth factors or their receptors can result in neoplastic transformation and altered development (12). There are several mechanisms by which growth factor receptors can be rendered transforming. Retroviral transduction of proto-oncogenes can result in truncation and mutation of the normal version of the gene. This has been shown for the epidermal growth factor receptor (EGFR) (v-*erbB*) (18), colony-stimulating factor 1 receptor (CSF-1R) (v-*fms*) (68), and *ros* (v-*ros*) (52) genes. Overexpression of an otherwise normal receptor, with or without the concomitant application of ligand, can also result in neoplastic transformation, as shown for the insulinlike growth factor 1 receptor (38), EGFR (15, 77), CSF-1R (65), *eph* (51), and *neu* (16) genes. Furthermore, structural rearrangement, as seen with *ret*, *trk*, and *met* (24, 50, 59), may also activate the transforming capacity of receptor kinases.

In addition to the transforming activity of altered or overexpressed receptor tyrosine kinases, studies have demonstrated that the normal cellular homologs of these receptors function to regulate cell growth and differentiation. Examples include the *trk* receptor kinase originally isolated by genomic transfection of human colon carcinoma DNA into NIH 3T3 cells (50). Although *trk* is not generally involved in colon cancer, recent work has demonstrated that this gene encodes a subunit of the nerve growth factor receptor which plays a critical role in neural development (39, 40). Furthermore, the *met* proto-oncogene, originally isolated by transfection of 3T3 cells with genomic DNA from the tumorigenic human osteogenic sarcoma cell line MNNG-HOS (10), has been demonstrated to be the cell surface receptor for hepatocyte growth factor, a potential growth factor for a broad spectrum of cell types as well as a mediator of liver regeneration (7). In addition, CSF-1R mediates the pleiotropic effects of its cognate ligand, CSF-1. Together, these two molecules stimulate the proliferation and differentiation of cells of the macrophage lineage (68).

In an effort to determine genes involved in the progression of chronic myelogenous leukemia (CML) to acute-phase leukemia, we previously reported the identification of a transforming gene in the DNAs of two patients with CML (47). Molecular cloning and characterization indicate that this gene, which we term *axl* (from the Greek word anexelekto, or uncontrolled), is a receptor tyrosine kinase with a structure novel among tyrosine kinases. Our data indicate that the *axl* protein has tyrosine kinase activity and is capable of transforming NIH 3T3 cells. Furthermore, *axl*'s transforming capacity results from overexpression of *axl* mRNA rather than from structural mutation.

MATERIALS AND METHODS

Identification of a transforming gene in CML cells. Transfections and nude mouse tumorigenicity assays were performed as described previously (47). The cell lines AF6295 and AF3642 were derived from secondary nude mouse tumors arising from transfection of DNA from blast crisis and chronic-phase CML patients, respectively. Tumors were isolated from nude mice and digested in the presence of trypsin-EDTA. A portion of these cells was then plated in plastic tissue culture flasks.

Isolation of cosmid and cDNA clones. DNA fragments for cosmid cloning were generated by partial *Mbo*I digests of genomic DNA from a secondary nude mouse tumor cell line, AF6295. The restricted DNA fragments were size selected on sodium chloride gradients and cloned in the c2RB cosmid vector as previously described (4). Following ligation, recombinants were packaged with Gigapack Gold (Stratagene, La Jolla, Calif.) according to the manufacturer's recommen-

* Corresponding author.
† Present address: Department of Internal Medicine, Hematology, Universitaets Klinikum Rudolf Virchow, Spandauer Damm 130, D-W1000 Berlin, Germany.

EXHIBIT B

dations and used to infect *Escherichia coli* W46-4. The cosmid library was screened by using the Blur8 probe, which contains human *Alu* repetitive sequences (37), resulting in the identification of two *Alu*-positive clones, 1-1 and 4-2.

Unique human exon fragments were identified by differential screening of Southern blots of cosmid clones 1-1 and 4-2. Poly(A)$^+$ RNA from both AF6295 and untransformed NIH 3T3 cells was obtained as previously described (1). First-strand cDNA reactions were performed in a 50-μl final volume of 0.6 mM dCTP, 1 mM each dATT, dGTT, and dTTP, 10 mM dithiothreitol, 20 U of RNasin, 100 μg of random hexamers (Pharmacia, Piscataway, N.J.) per ml, 100 μCi of [^{32}P]dCTP (3,000 Ci/mmol; Dupont/NEN), 1× reverse transcriptase buffer (50 mM Tris [pH 8.3], 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 100 mg of bovine serum albumin [BSA] per ml, 20 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.), and 200 ng of poly(A)$^+$ mRNA. The mixture was incubated at 42°C for 45 min and denatured at 95°C for 5 min. Unincorporated label was separated on a Sepharose G-50 column. The resulting probes were used for Southern hybridization (1), using sheared and denatured mouse or human DNA at 100 μg/ml and salmon sperm DNA at 100 μg/ml for blocking. Southern blots were also hybridized with Blur8 to identify fragments from the cosmid clones containing *Alu* repeat sequences. A single 2.8-kb *Eco*RI-*Bam*HI fragment (pcc-1) in cosmid clone 1-1 was found to be free of *Alu* and mouse sequences while containing transcribed exons from the putative human transforming gene.

cDNA libraries were constructed in λgt10 by using poly(A)$^+$ RNA from AF6295. By using pcc-1 as a probe, several cDNAs were identified. Sequence analysis of these cDNAs indicated these clones were short (approximately 1 kb in size) and did not contain poly(A)$^+$ tails. A full-length cDNA, clone 1-4, was then isolated from a size-selected, oligo(dT)-primed cDNA library constructed as described previously (26) except that 4 μg of poly(A)$^+$ RNA was reversely transcribed by using Superscript Moloney murine leukemia virus (Bethesda Research Laboratories, Gaithersburg, Md.). After second-strand synthesis, cDNA products were fractionated on a 0.8% agarose gel in 1× TAE (0.04 M Tris-acetate, 0.002 M EDTA). Products in the range of 2.7 to 6 kb were electrophoresed onto NA-45 paper (Schleicher & Schuell, Keene, N.H.) and then eluted twice with 250 μl of elution buffer (1 M NaCl, 50 mM arginine, free base). The eluate was extracted three times with phenol-chloroform (1:1) and ethanol precipitated. cDNA products were ligated in *Eco*RI-cut λgt10 arms (Stratagene), packaged into phage heads with Gigapack Gold (Stratagene), and then plated on *E. coli* C600Hfl. Recombinants were screened with an *axl*-specific cDNA. Positive clones were subcloned into pBluescriptII-KS (Stratagene) for subsequent sequence analysis.

Normal *axl* cDNA clones were isolated as described above, using a size-selected cDNA library constructed from poly(A)$^+$ RNA from the normal human diploid fibroblast cell line IMR-90. Recombinant phage were screened by using a polymerase chain reaction (PCR) probe generated from clone 1-4 DNA, using primers PJA-14 (bp 2090 to 2109; GCCCACTCAGATGCTAGTGA) and PJA-17 (bp 2569 to 2588; CAAGGCCTTCAGTGTGTTCT). Positive clones were subcloned into pBluescriptII-KS for subsequent sequence analysis.

DNA sequencing. Complete sequence from both strands was determined by the dideoxy termination method (66), using the modified T7 polymerase (Sequenase; United States Biochemical, Cleveland, Ohio). Overlapping fragments for sequence analysis were generated by digestion with exonuclease III (31) or by priming with sequence-specific oligonucleotides. Sequences were compared with the BESTFIT DNA sequence analysis program (University of Wisconsin Genetics Computer Group [14]).

RNA-PCR analysis of *axl* expression. RNA (100 ng of mRNA or 1 μg of total RNA) was denatured at 95°C for 3 to 5 min in the presence of 100 pmol of random hexamers (Pharmacia) and cooled on ice for 2 min. The RNA was reverse transcribed in a 20-μl final volume of 1× PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 625 μM each deoxynucleoside triphosphate (dNTP), 20 U of RNasin (Promega), 10 mM dithiothreitol, and 200 U of Superscript Moloney murine leukemia virus reverse transcriptase. The reaction mixture was incubated for 10 min at room temperature, 45 min at 42°C, and 5 min at 95°C. One microliter of cDNA product was PCR amplified in a 50-μl final volume of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.001% gelatin, 0.5 μM each primer, and 1.25 U of *Taq* polymerase (Promega) for 30 cycles under the following conditions: 5 min at 94°C, 1 min at 55°C, 1.5 min at 72°C for 1 cycle; 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C for 28 cycles; 1 min at 94°C, 10 min at 60°C. Differential PCR (57) was performed, using α-actin as a reference. Ten percent of this reaction mixture was electrophoresed on a 12% polyacrylamide gel and stained with ethidium bromide. Primer sequences are as follows: PJA-2 (bp 1847 to 1866), GGTGGCTGTGAAGACGATGA; PJA-3 (bp 2149 to 2130), CTCAGATACTCCATGCCACT; PJA-8 (bp 1320 to 1339), GAGGTGACCCTGGAGCTGCA; JB-2 (bp 1490 to 1471), AGGAGTTGAAGGTCCCTTCA; 5′ α-actin, CCTTCCTGGGCATGG AGTCCT; and 3′ α-actin, GGA GCAATGATCTTGATCTTC.

Chromosomal localization and fluorescence in situ chromosomal hybridization. A Southern blot of DNA from human-hamster somatic cell hybrids (BIOS, New Haven, Conn.) was probed with random-primer-labelled cDNA clones 12-1 and 1-4 and genomic clone pcc-1 under conditions recommended by the manufacturer. The filter was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) for 10 min at room temperature, once in 1× SSC–1.0% SDS for 15 min at 65°C, and twice in 0.1× SSC–1.0% SDS for 15 min at 65°C and then exposed overnight at −70°C.

For in situ hybridization, human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. The *axl* probe was cosmid 1-1 containing a 41-kb genomic fragment cloned in the cosmid vector c2RB. The procedure used for fluorescence in situ hybridization is a modification of the method described by Lichter et al. (45). Biotin-labelled probes were prepared by nick translation using Bio-11-dUTP (Enzo Diagnostics, New York, N.Y.). Chromosomal DNA was denatured by immersion of the slides in 70% formamide–4× SSC (pH 7.0) at 70°C for 2 min. Prior to hybridization, unlabelled, sonicated, total human genomic DNA (150 mg/ml) was added to the hybridization mixture (50% formamide, 1× SSC, 10% dextran sulfate, pH 7.0) containing the labelled probe (50 to 100 ng). The hybridization mixture was heated at 75°C for 5 min and then incubated at 37°C for 5 to 10 min to promote partial reannealing. After hybridization, the slides were washed in 50% formamide–4× SSC (three washes, 5 min each) at 40°C and then in 4× SSC at 40°C (three washes, 3 min each).

Detection reagents were prepared in 4× SSC–0.1% Triton X-100–1% BSA, and the washes were performed in 4× SSC–0.1% Triton X-100 at 40°C (three washes, 3 min each).

For the detection of the labelled probe, slides were incubated with fluorescein isothiocyanate-conjugated avidin (5 mg/ml; Vector Laboratories, Burlingame, Calif.) at 37°C for 30 min and then washed. Metaphase cells were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (200 ng/ml in 2× SSC, 5 min, room temperature; Sigma, St. Louis, Mo.) and mounted in 20 mM Tris-HCl (pH 8.0)–90% glycerol containing 2.3% DABCO antifade (Sigma). The slides were examined under a Zeiss microscope equipped with epifluorescence optics.

Hybridization analysis. Northern (RNA) and Southern analyses were performed according to standard protocols (1). RNAs were isolated from cell lines by the method of Glisin et al. (23) or Chomczynski and Sacchi (9). Radiolabelled probes were generated by the random-priming technique or PCR (21, 35), using the primer pairs PJA-2 and PJA-3 (see above); PJA-8 (see above) and PJA-9 (bp 1890 to 1871; CTGACCTCGTGCAGATGGCA); PJA-11 (bp 646 to 627; TGAGCTTGGCAGCTCAGGTT) and PJA-12 (bp 248 to 267; GCAGGCTGAAGAAAGTCCCT); and PJA-14 (bp 2090 to 2109; GCCCACTCAGATGCTAGTGA) and PJA-17 (bp 2569 to 2588; CAAGGCCTTCAGTGTGTTCT).

Transfection and tumorigenicity assays. A 100-mm dish containing approximately 5×10^5 NIH 3T3 cells was fed with fresh medium 4 to 24 h prior to transfection. Five micrograms of plasmid DNA was precipitated with 3 M sodium acetate in the presence of 30 μg of high-molecular-weight salmon sperm DNA in a 100-μl volume. The resulting precipitate was resuspended in 450 μl of H_2O and incubated overnight at 37°C with gentle rocking. On the following day, 50 μl of 2.5 M $CaCl_2$ was added dropwise while air was bubbled through a plugged pipette. This solution was then added dropwise to 500 μl of 2× HBS (280 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 1.5 mM $Na_2HPO_4 \cdot 12H_2O$, pH 7.05) while air was bubbled through a plugged pipette. This solution was allowed to stand for 20 to 30 min at room temperature without agitation. The fine precipitate was then exposed to 3T3 cells for 8 h at 37°C. The medium was removed, and 2 ml of 15% (vol/vol) glycerol in phosphate-buffered saline (PBS) was added to cells for 90 s. Cells were washed with PBS and fed with fresh medium. After 24 to 48 h, cells were split 1:4 into 100-mm dishes containing G418 (600 μg/ml; total drug). Emergent colonies were then pooled to produce the nonclonal cell lines used in subsequent analysis (TF14A and TF14B transfected with the axl^+ sense construct; TF15A and TF15B transfected with the axl^+ antisense construct; TF16A and TF16B transfected with the pLXSN vector).

For analysis of transformation, half of the pooled G418-resistant cells were passaged once, grown to confluence, and scored for focus formation after 2 to 3 weeks. The remainder of these cells were expanded and injected into the flanks of athymic nude mice at concentrations of 1×10^5 and 5×10^5 cells per site. Tumor formation was scored 50 days after injection.

Baculovirus expression. The baculovirus transfer vector pBlueBac (Invitrogen, San Diego, Calif.) contains a β-galactosidase expression cassette which permits recombinant plaque screening by addition of the chromagenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 0.15 mg/ml) to the agarose overlay. The cDNA insertion site of pBlueBac is an *Nhe*I site. An *axl* cDNA segment with full-length coding sequence and *Nhe*I-compatible ends was produced by cutting the *axl* cDNA clone 1-4 at the translation start site with *Nco*I. The resulting 3′ overhangs were filled in with the Klenow fragment of DNA polymerase I plus dNTPs. After heat inactivation, the fragment was digested with *Eco*RI, ligated into *Sma*I-*Eco*RI-digested pBluescriptII-KS plasmid, and then used to transform *E. coli*. The resulting plasmid contains an *Spe*I site 12 bp upstream of the *axl* ATG start site and an *Xba*I site 265 bp downstream of the translation stop site. This *Spe*I-*Xba*I fragment was cloned into *Nhe*I-digested pBlueBac. The resulting *axl* transfer vector and wild-type *Autographa californica* nuclear polyhedrosis virus DNA were cotransfected into *Spodoptera frugiperda* Sf9 cells (72). Subsequent plaque selection yielded the recombinant virus *axl*-BlBac. Positives were evaluated for the presence of *axl* sequences with PCR, using primer pair PJA-5 (bp 951 to 970; TCAGACGATGGGATG GGCAT) and PJA-6 (bp 1006 to 1025; CACGGATG CTTGC GAGGTGA) or PJA19 (bp 2755 to 2774; ATGTCCTCTGC CCTTCCACA) and PJ-7 (bp 2827 to 2843; TCAGGCACCA TCCTCCT).

Immunoblot analysis of *axl* expression. For analysis of baculovirus expression, Sf9 cells were grown at 27°C in monolayers (2×10^6 cells per 60-mm dish) in TMN-FH medium (Invitrogen) and infected with wild-type or *axl*-BlBac virus at a multiplicity of infection of greater than 10. After 1, 2, and 3 days, the dishes were placed on ice and the cell monolayers were rapidly rinsed with PBS, lysed, and then scraped in 0.3 ml of RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 1% sodium deoxycholate, 0.1% SDS, 100 μM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 mM *p*-nitrophenylphosphate). After addition of 0.15 ml of 3× Laemmli gel sample loading buffer (30 mM Tris-HCl [pH 7.8], 9% SDS, 15% glycerol, 6% 2-mercaptoethanol, 0.05% bromophenol blue) and boiling for 5 min at 100°C, 50-μl aliquots of the samples were electrophoresed on 9% SDS-polyacrylamide gels. The proteins were electrotransferred from the gel onto a polyvinyl difluoride membrane (Immobilon-P; Millipore, Bedford, Mass.). The membrane was treated for 30 min with 150 mM NaCl–10 mM Tris-HCl (pH 7.5)–0.05% Tween-20 (TBST) containing 1% BSA. The blot was then incubated for 1 h with antiphosphotyrosine monoclonal antibody PT-66 (Sigma) diluted 1:2,000 in TBST. Using a duplicate blot, 1 mM phenylphosphate was included with the antiphosphotyrosine antibody to block specific binding to phosphotyrosine. The blot was rinsed three times for 5 min each time with TBST and then incubated with anti-mouse immunoglobulin G-alkaline phosphatase conjugate (A-5153; Sigma) diluted 1:2,000 in TBST for 45 min. After three rinses in TBST, the blot was incubated with the chromagenic substrates nitroblue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate (0.165 mg/ml) in alkaline buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 10 mM $MgCl_2$) for 5 to 20 min. Color development was stopped by rinsing with deionized water. Analysis of tyrosine-phosphorylated proteins in the *axl*-transformed 3T3 cells (see Fig. 7B) was performed according to published protocols (53).

The polyclonal anti-*axl* antiserum was prepared by immunizing rabbits with gel-purified baculovirus-expressed *axl* protein. The antigen in the form of a Coomassie-stained gel slice was processed and administered in primary and (after 1 month) boost injections (28). The anti-*axl* serum was collected 25 days following the boost injection. Immunoblot analysis was performed with the anti-*axl* antiserum and preimmune serum diluted 1:3,000 in TBST. Anti-rabbit immunoglobulin G-alkaline phosphatase conjugate was used as the secondary antibody.

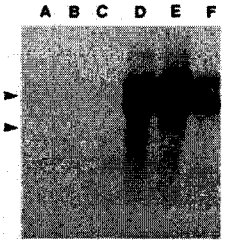

FIG. 1. Expression of *axl* in nude mouse tumors. Poly(A)$^+$ RNA from various nude mouse tumors was fractionated on a formaldehyde-agarose gel, transferred to nylon, and probed with pcc-1 labelled by random priming (21). Lanes: A, untransformed NIH 3T3 cell line; B, EJ-*ras*-transformed NIH 3T3 cell line 1; C, EJ-*ras*-transformed NIH 3T3 cell line 2; D, AF6295-1; E, AF6295-2; F, AF3642. Samples in lanes D and E are from two different passages of the nude mouse tumor explant cell line derived from transfections with DNA from a CML blast crisis patient. AF3642 is another explant cell line derived from transfection with DNA from a CML chronic-phase patient. The single band seen in lanes A through C represents the normal mouse homolog of *axl*. Arrowheads indicate the positions of 28S and 18S rRNAs.

RESULTS

Cloning of the transforming *axl* gene. In our previous study, using a sensitive NIH 3T3 transfection-tumorigenicity assay, we identified a transforming gene in the DNA from the peripheral leukocytes of two CML patients that lacked *ras* mutations (47). The resulting tumors arising from secondary transfection of tumor DNA gave rise to two explant tumor cell lines (AF6295 and AF3642) which harbored the same unique transforming human sequences. Several cosmid clones bearing human *Alu* repeats were isolated from a cosmid library derived from the DNA of one such explant tumor cell line, AF6295 (see Materials and Methods). Exon mapping identified a genomic fragment, pcc-1, that contained transcribed human sequences but was devoid of *Alu* repeats and mouse exons. Using pcc-1 as a probe on Northern blots, two *axl* mRNA species of 5.0 and 3.4 kb were identified in the explant 3T3 cell lines from the CML transfectants but not in untransformed NIH 3T3 cells or in 3T3 cells transformed by a mutant H-*ras* oncogene (Fig. 1). Using pcc-1 as a probe, several *axl*-specific cDNA clones were isolated from cDNA libraries derived from AF6295 poly(A)$^+$ RNA. All hybridized to the same 5.0- and 3.4-kb transcripts in the nude mouse tumor explant cell lines on Northern blot analysis. Sequence analysis of these cDNAs showed sequences in common with pcc-1. Since the original cDNA clones were short and did not contain poly(A)$^+$ tails, a size-selected cDNA library was screened to identify full-length cDNAs. Several clones were isolated and found to contain 3.2-kb inserts consistent in size with one of the two *axl* mRNA species. One of these clones, 1-4, was subcloned and sequenced.

Sequence analysis indicates that *axl* is a novel receptor tyrosine kinase. The sequence of clone 1-4 predicted a protein of 894 amino acids with two in-frame methionine residues that are candidate initiating amino acids based on Kozak consensus rules (Fig. 2) (43). Furthermore, an in-frame termination codon lies 170 bp upstream from the first methionine codon. This methionine marks the beginning of a potential signal sequence of 32 amino acids (78). A search of the EMBL, GenBank, and NBRF protein data bases indicated that the *axl* protein is a novel protein tyrosine kinase with significant homology to many known kinases, in particular *eph*, *eck*, *elk*, *ros*, *trk*, insulin receptor, insulin-related receptor, insulinlike growth factor 1 receptor, and sevenless (4, 19, 32, 44, 46, 49, 52, 69, 75) (Fig. 3B and C). The predicted peptide at amino acids 543 to 569 contained the consensus sequence GxGxxG...(15 to 20 amino acids) ...AxKxM, which functions as the Mg^{2+}-ATP binding site for tyrosine kinases (27). At approximately 100 amino acids carboxyl terminal to the Mg^{2+}-ATP binding domain in *axl* lies the sequence DLAARN...(34 amino acids)...PVKWI AIE, which resembles but is distinct from a consensus sequence specific to tyrosine kinases, DLAARN...P(I/V)(K/R)W(T/M)APE (27). Furthermore, there are two sites at amino acids 779 and 821 in the carboxyl terminus of *axl* that are similar to the consensus sequence (E/D)(E/D)(E/D)(E/D)(E/D)Y(M/V)PMXX, where Y is phosphotyrosine, for a phosphatidylinositol (PtdIns) 3-kinase binding site (8). These observations suggest that *axl* is a tyrosine kinase that interacts intracellularly with PtdIns 3-kinase.

Amino acids 448 to 472 in the *axl* protein comprise a hydrophobic region consistent with a transmembrane domain (Fig. 2 to 4). Within the putative extracellular domain, sequences (amino acids 224 to 428) amino terminal to the transmembrane region of the *axl* protein encode two fibronectin type III (FNIII) repeats (62, 71). Amino terminal to the FNIII repeats in *axl* (amino acids 37 to 212) are two immunoglobulinlike (IgL) repeats (79). Highly conserved cysteine and tryptophan residues (designated by asterisks in Fig. 3A) are characteristic of IgL domains. In addition, the extracellular domain of the *axl* protein contains six consensus sites for N-linked glycosylation, NxT/S, predicting that the mature *axl* protein is glycosylated. The juxtaposition of IgL and FNIII repeats in the extracellular region of the *axl* protein has not been reported for any known receptor tyrosine kinase, thus making the *axl* protein unique among this class of proteins.

Biologic activity of *axl*. Using cDNA clone 7-1 as a probe for Southern hybridization with nude mouse tumor DNAs, we detected human *axl* sequences in the appropriate primary and secondary tumors, indicating that *axl* was the transforming gene passaged with each cycle of transfection (data not shown).

To confirm *axl*'s transforming potential, we constructed retroviral expression vectors containing the full-length *axl* cDNA (clone 1-4). In these constructs, transcription of *axl* is under the control of a Moloney murine sarcoma virus long terminal repeat, whereas the neomycin phosphotransferase gene, which confers resistance to the drug G418, is driven by a simian virus 40 promoter (55). NIH 3T3 cells were transfected with the *axl* sense (pL1-4S) or antisense (pL1-4AS) construct or vector alone (pLXSN) and then assayed for transformation in a standard focus formation assay (i.e., without G418 selection). No foci were observed in the focus formation assay. Furthermore, colonies arising after G418 selection did not appear transformed. However, when the G418-resistant colonies were passaged once and allowed to reach confluence, foci emerged in the cells transfected with pL1-4S (*axl*$^+$ sense construct) but not pL1-4AS (*axl*$^+$ antisense construct) or pLXSN (vector) (Fig. 5). This finding suggests that *axl* alone is necessary but insufficient for transformation in this system and that either a second genetic event or selection for cells overexpressing *axl* is required for *axl* to be transforming. Northern analysis was performed on RNA isolated from the initial pooled G418-resistant cells (cells transfected with the sense *axl*, TF14A and TF14B; cells transfected with the anti-sense *axl*, TF15A

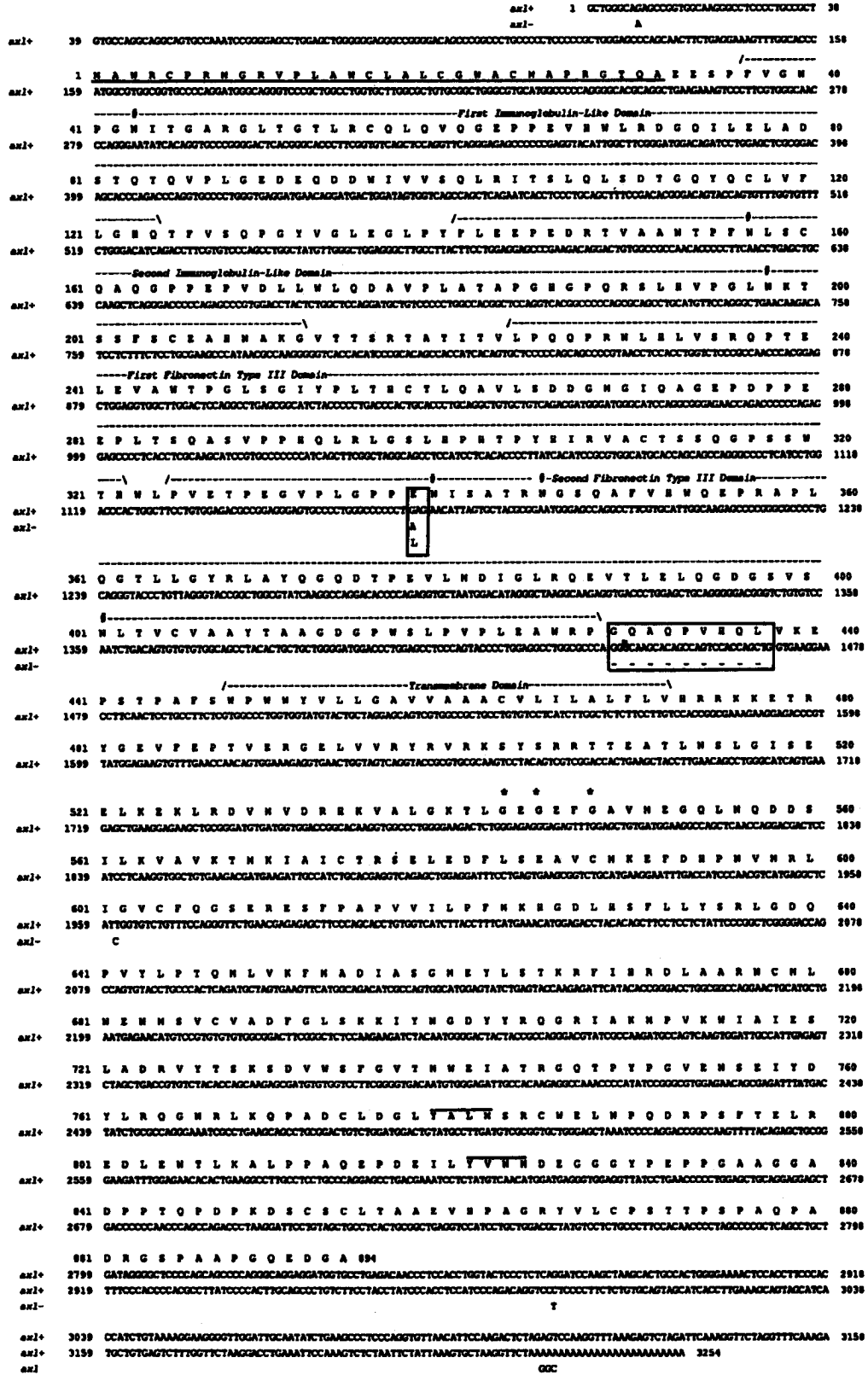

FIG. 2. Sequences of *axl* cDNAs and predicted peptides. Shown are the complete nucleotide and amino acid sequences of clone 1-4 (axl^+) isolated from the nude mouse tumor cell line AF6295, with the predicted peptide sequence on the top line. Only the nucleotide differences between clone 6-2 (axl^-) and clone 1-4 are marked below the 1-4 nucleotide sequence. Differences in the predicted peptide sequence of clone 6-2 are indicated below the 6-2 nucleotide sequence with a boxed area highlighting these differences. The minus signs indicate sequences not present in clone 6-2. Underlined area, signal peptide; #, potential N-linked glycosylation sites; *, conserved residues of the ATP-binding site marking the beginning of the kinase domain; solid overlined area, putative autophosphorylation sites which potentially bind PtdIns 3-kinase. A stop codon (not shown) lies 170 bases upstream of the first methionine and was present in an overlapping cDNA clone which was 270 bp more 5' of the beginning of 1-4.

and TF15B; cells transfected with the vector alone, TF16A and TF16B) and transformed foci cloned from cells transfected with the sense *axl* cDNA (subclones TF14B1, -14B2, -14B3, -14B7, and -14B10). Expression of the human *axl* gene was seen in cells transfected by sense (TF14A and -B) and antisense (TF15A and -B) constructs, albeit at low levels. However, the *axl*-transformed subclones expressed high levels of *axl* RNA (Fig. 6A). Thus, the second event in *axl* transformation appears to be a selection for cells expressing increased levels of *axl* message, indicating that overexpression of *axl* is necessary for 3T3 transformation.

We next tested the tumorigenic capacity of cells transfected with the genomic *axl* gene (AF6295) or *axl* cDNA (clones TF14B1, -14B2, -14B3, -14B7, and -14B10). Injection of either AF6295 or clone TF14B1, TF14B2, TF14B3, TF14B7, or TF14B10, all of which express high levels of *axl* message, resulted in the emergence of tumors within 9 days (Table 1). There appeared to be a rough correlation between a short tumor latency with increased expression of *axl* RNA and protein, further supporting the notion that *axl* overexpression drives 3T3 transformation. In contrast, the initial *axl* transfectants (TF14A, TF14B, TF15A, and TF15B), vector-transfected NIH 3T3 cells, and untransformed NIH 3T3 cells did not form tumors within an incubation period of 50 days except for one of the clones transfected with vector alone (TF16A). This clone formed a tumor after 43 days and therefore represents background tumor formation.

Tyrosine kinase activity of *axl*. To test whether *axl* is a tyrosine kinase, we analyzed the phosphotyrosine profile of cells transformed by either genomic *axl* or an *axl* cDNA. Using an antiphosphotyrosine antibody, a novel 140-kDa tyrosine-phosphorylated protein was seen only in cells transfected with *axl* and not untransformed or *ras*-transformed NIH 3T3 cells (Fig. 6B). Furthermore, the level of pp140 correlated with the level of *axl* RNA in these cells (Fig. 6).

To further test the tyrosine kinase activity of *axl*, recombinant *axl* protein was expressed in Sf9 insect cells, using the baculovirus expression system (Fig. 7). In a Coomassie-stained polyacrylamide gel of cellular proteins, a novel protein of approximately 104 kDa was induced in cells infected with a recombinant baculovirus containing *axl* cDNA (1-4) which was not present in wild-type-infected or uninfected cells (Fig. 7A). The molecular size of this recombinant protein corresponds roughly to the predicted size (95 kDa) of the unglycosylated *axl* protein minus the signal peptide as deduced from the amino acid sequence by using the PEPTIDESORT program (Genetics Computer Group of the University of Wisconsin [14]). Using an antiphosphotyrosine monoclonal antibody in Western immunoblot analysis, we detected a similar 104-kDa protein in *axl*-baculovirus-infected cells but not wild-type baculovirus-infected or uninfected cells (Fig. 7B). In addition, we detected a 120-kDa protein which we suspect represents a partially glycosylated form of the *axl* protein. As further evidence that the *axl*-baculovirus-induced bands represent actual tyrosine-phosphorylated proteins, the immunoreactive bands were abolished by the inclusion of 1 mM phenylphosphate with the antiphosphotyrosine antibody (Fig. 7B, lane 9). These data support the conclusion that the *axl* protein is a tyrosine kinase. Since insect cells do not glycosylate proteins to the same extent as do mammalian cells, we suspect that the 140-kDa phosphoprotein seen in *axl*-transformed 3T3 cells and the 120-kDa phosphoprotein in *axl*-infected Sf9 cells most likely represent fully processed and partially processed forms of *axl*, respectively. In support of this idea, we are able to detect 140- and 120-kDa proteins in the *axl*-transformed cell line AF6295 and 120- and 104-kDa proteins in *axl*-baculovirus-infected Sf9 cells, using a recently isolated polyclonal anti-*axl* antibody in immunoblot analysis of cellular proteins (Fig. 7C).

Cloning and characterization of c-*axl*. Many oncogenes have been shown to be activated by genomic alterations (24, 50, 59, 63, 73). We investigated whether rearrangements or deletions of *axl* were present in the original patient DNAs from which *axl* was isolated as compared with DNAs from normal peripheral leukocytes and from the nude mouse tumor lines harboring the human *axl* oncogene. By using probes corresponding to the kinase, transmembrane, and extracellular domains of the cloned *axl* cDNA in Southern blot analyses, no rearrangements of *axl* were detected in the DNAs from either the original CML patients or the nude mouse tumors as compared with DNAs from normal blood (data not shown). Furthermore, Northern blot analysis of *axl* transcripts from AF6295 and several normal and transformed cell types which also express *axl* mRNA (A549, A431, IMR-90, HeLa, and BG-9) revealed the identical 3.4- and 5.0-kb transcripts, making rearrangements and major deletions unlikely (Fig. 8A).

To determine the mechanism by which *axl* was activated, we cloned cDNAs of the normal *axl* homolog (c-*axl*). Using RNA from the normal human fibroblast IMR-90, we cloned a normal *axl* cDNA (clone 4-9) which appears identical to the transforming clone, 1-4. However, another cDNA clone isolated from the normal fibroblast library, 6-2, predicted an altered version of *axl* which lacked amino acids 429 to 437 (GQAQPVHQL) and contained a G-to-A transition at base 1170, resulting in the substitution of a leucine for a glutamic acid (Fig. 2). We will refer to the predicted *axl* transcript from 1-4 as axl^+ and that from 6-2 as axl^-. Reverse PCR analysis of RNA with oligonucleotide primers flanking amino acids 429 to 437 showed both species of *axl* message present in the nude mouse tumor cell lines as well as other human cell lines and a primary human fibroblast, suggesting that axl^+ and axl^- are splice variants from the same gene. However, the axl^+ species was overexpressed relative to the axl^- species at an average ratio of 17:1 in the nude mouse tumor cells lines; in comparison, normal human fibroblasts exhibited a ratio of 2:1 (Fig. 9). To test the transforming capacity of the *axl* cDNA, we expressed clone 6-2 in NIH 3T3 cells by using the pLXSN retroviral expression vector.

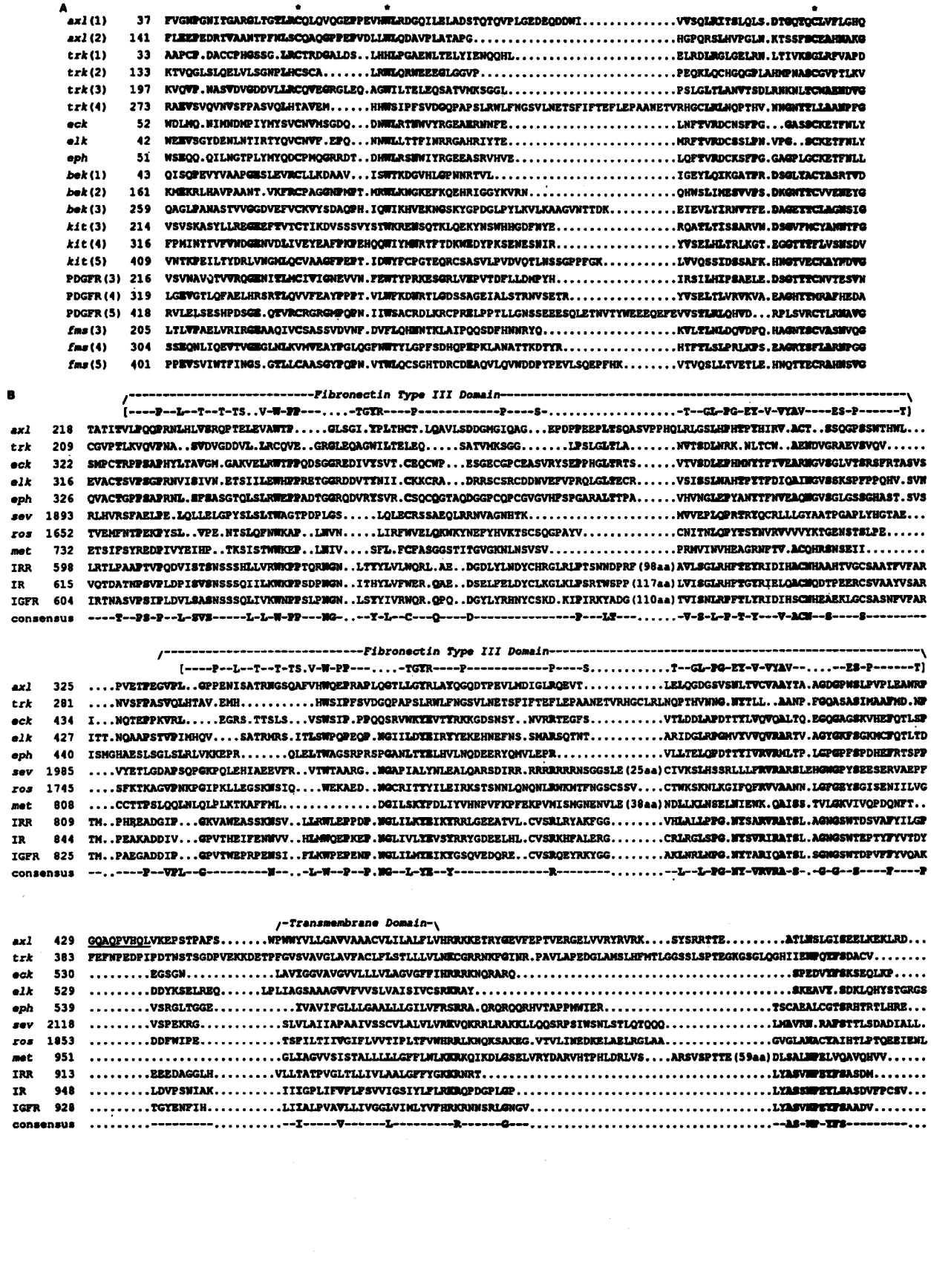

FIG. 3. Multiple amino acid sequence alignment of the *axl* protein with other receptor tyrosine kinases. Sequences were aligned by eye according to structural similarities. Amino acid positions numbered from the initiation methionine are shown at the beginning of each sequence. Gaps introduced for optimal alignment are designated by dots. (A) Comparison of IgL domains. Numbers in parentheses indicate which repeat was used in the alignment. Amino acids present in six or more sequences at a given position are in bold print. Asterisks indicate highly conserved amino acids. (B) Comparison of FNIII repeats. Shown above the sequence alignment is a consensus for FNIII domains representing residues present in at least 7 of the 15 FNIII repeats contained in bovine fibronectin (71). Below the alignment is a consensus sequence generated from the aligned receptor tyrosine kinases; residues present in five or more of the aligned sequences are included in the consensus and are in bold print. The underlined sequence at amino acids 429 to 437 represents a differentially spliced exon of *axl* (see text). (C) Comparison of the tyrosine kinase region. The consensus sequence listed at the bottom of each group was derived from the aligned sequences. Parameters for bolding and the derivation for the consensus sequence are as described for panel B. *1 and *2 designate putative autophosphorylation sites which potentially bind PtdIns 3-kinase. Sequences are for human proteins except for *elk* (rat) and sevenless (*Drosophila melanogaster*). Sequence sources are as follows: *trk* (49); *eck* (46); *elk* (44); *eph* (32); *bek* (17); *kit* (80); PDGFR, (25); *fms* (11); *sev* (3); *ros* (6); *met* (59a); IRR (insulin-related receptor) (69); IR (insulin receptor) (19); and IGFR (insulinlike growth factor 1 receptor) (75).

Using the same assay as with 1-4, expression of 6-2 also resulted in the emergence of foci after a single passage of G418-resistant colonies (Fig. 5). These data suggest that the activation of *axl*'s transforming capacity is due to overexpression and not to structural changes.

Expression of *axl*. Using both Northern blot and differential PCR analyses on cDNA (1, 57), variable expression of *axl* was seen in most human cell lines tested, including cell lines of epithelial, mesenchymal, and hematopoietic origins (Table 2; Fig. 8). However, AF6295 overexpresses *axl* relative to the other cell lines. In addition, *axl* was expressed in normal, nontransformed cells, including primary fibroblast and breast epithelium. The near-ubiquitous expression of *axl* suggests an important normal cellular function for this receptor kinase.

Chromosomal localization of *axl*. Various cDNA fragments of *axl* were hybridized to a Southern blot of DNAs from human-hamster somatic cell hybrids (BIOS, New Haven, Conn.) to determine the chromosomal position of *axl*. All results indicated that *axl* is located on human chromosome 19. To determine the location of the *axl* gene by an independent method, we used fluorescence in situ chromosomal hybridization of a biotin-labelled *axl* cosmid clone, 1-1, to normal human metaphase chromosomes. Of 25 cells exam-

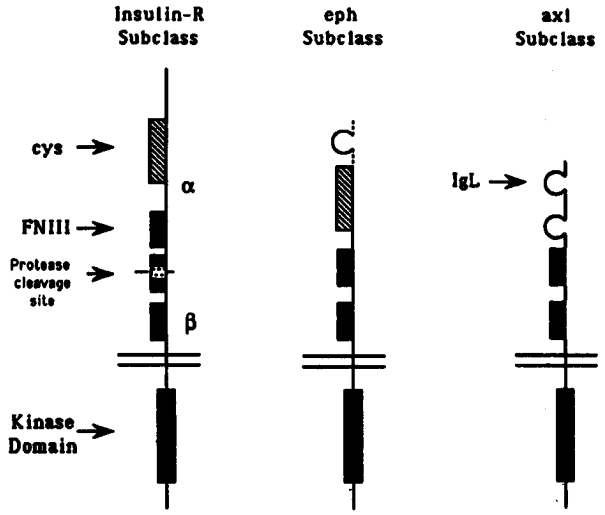

FIG. 4. Schematic representation of the various subclasses of the insulin receptor family. This classification is an extension of previously described classifications of receptor kinases (27, 76). The dashed lines in the *eph* family IgL repeat indicate that this repeat bears weak homology to IgL repeats (see Discussion).

ined, specific labelling was observed on one (4 cells), two (4 cells), three (11 cells), or all four (6 cells) chromatids of chromosome 19 homologs; the signal was localized to the long arm of this chromosome, at band q13.2 (Fig. 10). Similar results were obtained in a second hybridization experiment using this probe.

DISCUSSION

axl encodes a receptor tyrosine kinase with high amino acid similarity to the human *trk*, *eph*, *eck*, and *ros* proteins, insulin and insulin-related receptor, and insulinlike growth factor 1 receptor (Fig. 3 and 4). Novel sequence differences in the kinase domain [i.e., KWIAIE compared with the consensus (K/T)W(T/M)APE] set the *axl* protein apart from known kinases. Supporting the conclusion that *axl* encodes a tyrosine kinase, we found a novel 140-kDa tyrosine-phosphorylated protein in *axl*-transformed NIH 3T3 cells. Increased expression of *axl* RNA in these cells correlated with increased levels of pp140 and the tumorigenic potential of the transformed cells in nude mice. These results are consistent with the notion that pp140 represents a phosphorylated *axl* protein. Expression of *axl* in baculovirus resulted in the production of two novel tyrosine-phosphorylated proteins of 104 and 120 kDa which most likely represent unglycosylated and partially glycosylated forms of *axl*. The difference in molecular size can be explained by the fact that insect glycoproteins are processed differently from mammalian glycoproteins. Both insect and mammalian cells add a characteristic high-mannose oligosaccharide as the first step in N-linked glycosylation (34, 36). These glycoprotein intermediates are then processed to a trimannosyl core. Although mammalian cells further process this glycosylated intermediate by the addition of a variety of terminal sugar residues, no further processing takes place in insect cells (56). There-

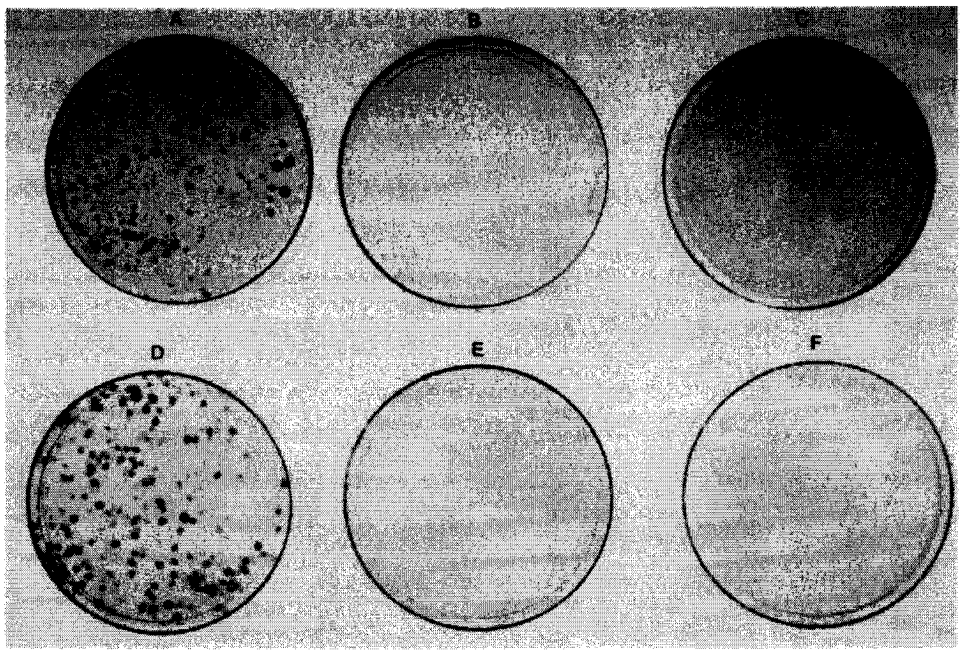

FIG. 5. Analysis of *axl*-transformed cells. NIH 3T3 cells were transfected with expression vectors containing *axl* in the sense or antisense orientation or vector alone and then selected with G418 (600 μg/ml; total compound). Emerging colonies from each transfection were pooled, passaged once, allowed to grow to confluence, and assayed for focus formation. Foci emerged after 10 to 14 days in culture. Cells were then stained with crystal violet (0.2% in H_2O). Samples are as follows: (A) cells transfected with cDNA clone 1-4 in the sense orientation; (B) cells transfected with cDNA clone 1-4 in the antisense orientation; (C) cells transfected with pLXSN vector; (D) cells transfected with cDNA clone 6-2 in the sense orientation; (E) cells transfected with cDNA clone 6-2 in the antisense orientation.

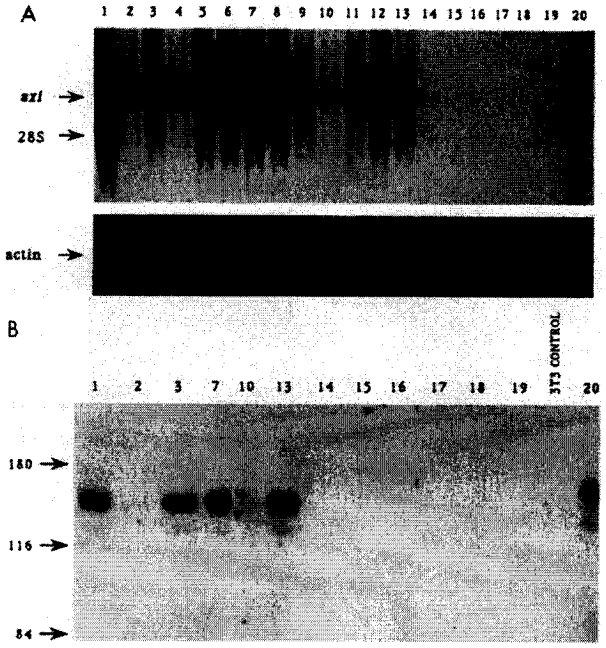

FIG. 6. Analysis of *axl* expression in transfected NIH 3T3 cells. (A) Northern analysis. Fifteen micrograms of total RNA was fractionated on a formaldehyde-agarose gel, transferred to nylon, and probed with a PCR probe generated with primers PJA-14 and PJA-17 (see Materials and Methods). Loading was normalized by probing with an actin probe generated as described above with actin-specific primers. Lanes: 1, TF14B1; 2, TF14B2; 3, TF14B3; 4, TF14B4; 5, TF14B5; 6, TF14B6; 7, TF14B7; 8, TF14B8; 9, TF14B9; 10, TF14B10; 11, TF14B11; 12, TF14B12; 13, TF14B, passage 7; 14, TF14A; 15, TF14B; 16, TF15A; 17, TF15B; 18, TF16A; 19, TF16B; 20, AF6295. (B) Western analysis. Equivalent amounts of protein were loaded on an 8% polyacrylamide gel, transferred to nylon, and then immunoblotted with an antiphosphotyrosine antibody (53). Lanes correspond to those on panel A. Sizes are indicated in kilodaltons on the left.

fore, we suspect that the novel 140-kDa phosphoprotein in *axl*-transformed 3T3 cells is a phosphorylated, fully glycosylated form of *axl*. This conclusion is further supported by immunoblot experiments with an anti-*axl* polyclonal antibody which reacts with what appear to be the same 120- and 140-kDa proteins seen in the antiphosphotyrosine Western blots.

The kinase domain of *axl* contains two PtdIns 3-kinase consensus binding sites (Fig. 2). PtdIns 3-kinase was originally found to complex with middle t antigen of polyomavirus and $pp60^{c\text{-}src}$. Attention focused on PtdIns 3-kinase when it was found that association of this enzyme with transforming tyrosine kinases correlated with their kinase activity and their ability to transform cells. Various other receptors, including the platelet-derived growth factor beta receptor (PDGFβR), CSF-1R, and EGFR, have been shown to associate with PtdIns 3-kinase. In addition, receptor kinases have been shown to complex with the *ras* GTPase-activating protein and, in the case of EGFR and PDGFβR, with PtdIns-specific PLC-γ (reviewed in reference 8). These protein complexes are believed to play a central role in receptor tyrosine kinase signalling pathways and are formed by the binding of *src* homology regions (SH2) in proteins such as GTPase-activating protein to the phosphorylated receptor (8). Thus, the predicted *axl* peptide sequence suggests that *axl* may also complex with PtdIns 3-kinase.

Although similar to other receptor tyrosine kinases, the *axl* protein represents a novel subclass of receptor kinases based on the unique structure of the extracellular region that juxtaposes IgL and FNIII repeats. Between amino acids 224 and 428 lie two FNIII repeats (Fig. 2). This sequence motif was first identified as 60- to 100-amino-acid tandem repeats reiterated 15 times in fibronectin (71). A number of extracellular matrix proteins and receptors contain FNIII repeats, including neural cell adhesion molecules, the growth hormone/prolactin receptor family, the product of the *Drosophila* sevenless gene, and several receptorlike tyrosine phosphatases (4, 22, 29, 58, 61). In addition, we have found that a number of mammalian tyrosine kinases, including members of the insulin receptor family, also contain FNIII repeats. On the basis of homologies in the kinase domain and the presence of FNIII repeats, the *axl* protein appears most closely related to the insulin receptor family (Fig. 3). However, two copies of IgL repeats encompassing amino acids

TABLE 1. Summary of tumorigenicity results

Cell line	Transfected DNA	Tumor latency (days)[a]	RNA *axl* level[b]	pp140 level[b]
AF6295	Genomic *axl*	3	+++++	++++
TF14A	*axl* sense cDNA	No tumors	+	−
TF14B	*axl* sense cDNA	No tumors	+	−
TF14B1[c]	*axl* sense cDNA	7	++++	+++++
TF14B2[c]	*axl* sense cDNA	6	+++	+
TF14B3[c]	*axl* sense cDNA	7	++++	++++
TF14B7[c]	*axl* sense cDNA	4	+++++	++++
TF14B10[c]	*axl* sense cDNA	8	+++	+
TF14B (passage 7)[d]	*axl* sense cDNA	7	++++	+++++
TF15A	*axl* antisense cDNA	No tumors	+	−
TF15B	*axl* antisense cDNA	No tumors	+	−
TF16A	Vector	43	−	−
TF16B	Vector	No tumors	−	−
NIH 3T3		No tumors	−	−

[a] Mice were observed for 50 days.
[b] +, qualitative difference in level; −, no expression.
[c] Subcloned foci from the TF14B transfectants.
[d] Cells from the parental transfectant, TF14B, which were pooled after foci emerged.

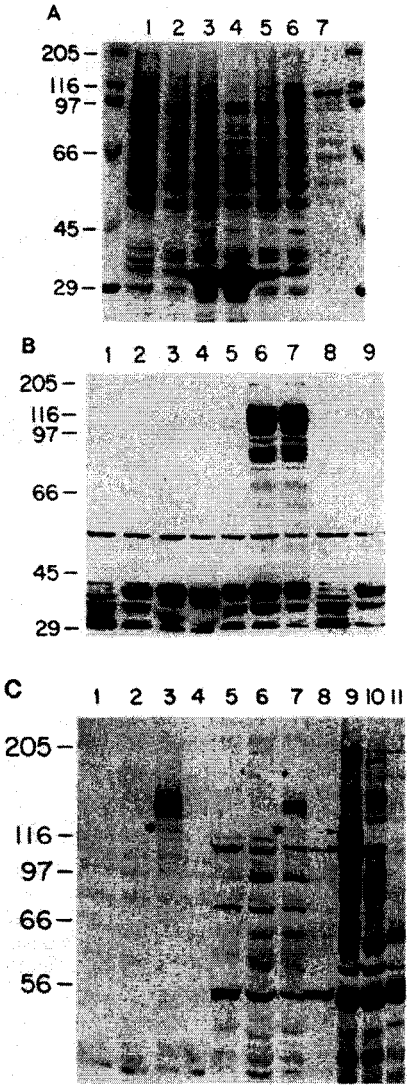

FIG. 7. Coomassie blue-stained gel (A) and antiphosphotyrosine Western blot analysis (B) of proteins from Sf9 cells infected with wild-type or recombinant *axl*-baculovirus. Total cell lysates were solubilized in sample buffer and then fractionated on a 9% polyacrylamide gel. (A) Lanes: 1, uninfected Sf9 cells; 2 to 4, cells infected with wild-type baculovirus for 1, 2, and 3 days, respectively; 5 to 7, cells infected with recombinant *axl*-virus for 1, 2, and 3 days, respectively. The arrow indicates the position of the recombinant *axl* protein. (B) Lanes: 1 to 7, same as in panel A; 8 and 9, identical to lanes 1 and 6 except that 1 mM phenylphosphate was added to block the antiphosphotyrosine antibody. (C) Western blot analysis of total cell lysates separated on a 7.5% polyacrylamide gel, using an antiphosphotyrosine monoclonal antibody in lanes 1 to 4 and anti-*axl* polyclonal antiserum in lanes 5 to 7, 9, and 10. Lanes: 1 and 5, untransformed NIH 3T3 cells; 2 and 6, *ras*-transformed 3T3 cells; 3 and 7, *axl*-transformed 3T3 cells (AF6295); 4, same as lane 3 except blocked with 1 mM phenylphosphate; 8, same as lane 7 except that rabbit preimmune serum was used as the primary antibody; 9, Sf9 cells infected for 2 days with recombinant *axl*-baculovirus; 10, Sf9 cells infected for 2 days with wild-type baculovirus; 11, same as lane 9 except that preimmune serum was used as the primary antibody. Asterisks indicate partially processed pp120 *axl* protein. Arrows indicate pp120 and pp104 *axl* proteins in the *axl*-baculovirus-infected cells. Sizes are indicated in kilodaltons on the left.

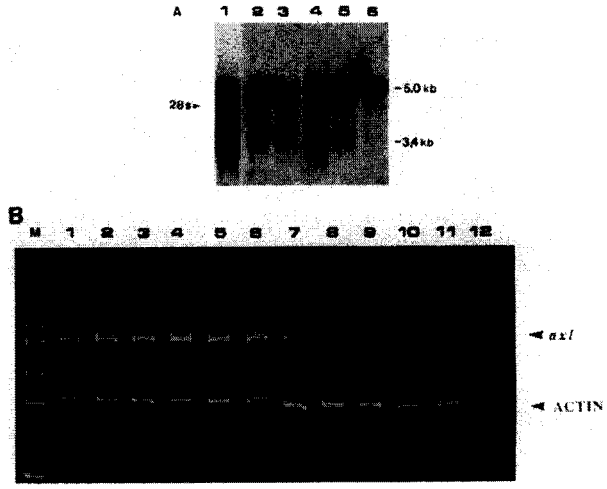

FIG. 8. Analysis of *axl* expression. (A) Northern analysis. The assay was performed as for Fig. 1 on 2 μg (lanes 2 to 6) or 0.5 μg (lane 1) of poly(A)$^+$ RNA except that the filter was probed with a ^{32}P-labelled region of the kinase domain of *axl* generated by PCR (35). Exposure time for lane 1 was 1 day; lanes 2 to 6 were exposed for 7 days. The position of the 28S RNA is indicated. Lanes: 1, AF6295; 2, A549; 3, A431; 4, IMR-90; 5, HeLa; 6, BG-9. (B) PCR analysis of RNA isolated from various cell lines. Differential PCR (57) was performed, using α-actin as a reference gene for semiquantitative analysis of *axl* expression. The ethidium bromide-stained polyacrylamide gel indicates the presence of a 303-bp *axl* fragment corresponding to the kinase domain and a 201-bp α-actin band as reference. Lanes: M, *Hae*III-digested marker; 1, AF6295; 2, A549; 3, A431; 4, IMR-90; 5, HeLa; 6, BG-9; 7, K562; 8, LAM; 9, CML chronic-phase peripheral blood; 10, myelodysplastic syndrome peripheral blood; 11, normal peripheral blood 1; 12, no RNA.

37 through 212 are present amino terminal with respect to the FNIII repeats (Fig. 2). Homologous IgL domains have also been found in many extracellular matrix proteins and receptors involved in protein-protein interactions, such as neural cell adhesion molecules, PDGFβR, and the leukocyte common antigen-related protein (22, 29). The combination of IgL and FNIII repeats is seen in the neural immunoglobulin superfamily of cell adhesion proteins and the protein tyrosine phosphatases. The *axl* protein, however, is the first receptor tyrosine kinase identified which incorporates both types of repeats. Though the functions of IgL and FNIII domains are unclear, their presence in numerous cell adhesion molecules and receptors suggests a potential function for these domains in cell-cell interaction.

Receptor tyrosine kinases have been classified into families based on sequence similarity and structural characteristics (27, 76). The EGFR family contains two cysteine-rich repeats in the extracellular domain of a monomeric receptor. The insulin receptor family has a disulfide-linked, $\alpha_2\beta_2$ heteromeric structure and contains both a cysteine-rich domain and FNIII repeats in the extracellular region. The PDGFR/fibroblast growth factor receptor family has an extracellular domain that is characterized by the presence of IgL repeats and a kinase domain that is interrupted by various numbers of amino acids termed the kinase insert. A number of kinases, including the *trk*, *eph*, and recently isolated *eph*-related *eck* and *elk* kinases, have not been incorporated in this classification scheme (44, 46). A detailed amino acid sequence comparison of these receptor kinases

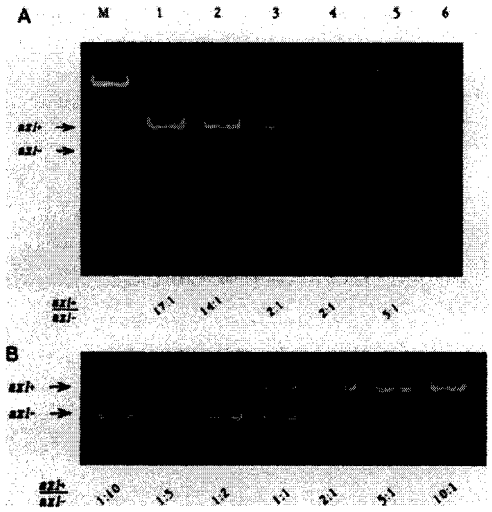

FIG. 9. RNA-PCR expression analysis of *axl* isoforms. (A) Samples were analyzed for expression of the axl^+ or axl^- species, using primers PJA-8 and JB-2 (see Materials and Methods), which flank the 27-bp insertion shown in Fig. 2. Lanes: 1, AF6295; 2, AF3642; 3, IMR-90; 4, A431; 5, K562 treated with 5 nM 12-*O*-tetradecanoylphorbol-13-acetate; 6, negative control. (B) Different molar ratios of cDNA clones 1-4 (axl^+) and 6-2 (axl^-) were mixed together and PCR amplified (see Materials and Methods) with primers PJA-8 and JB-2 to assess the relative intensities of the different species at various concentrations. A fraction of the product (1.5 μl) was electrophoresed on a 12% polyacrylamide gel and stained with ethidium bromide. Densitometry was performed to generate a standard curve used for comparison with samples in panel A.

TABLE 2. Summary of *axl* expression

Sample	Cell line	Expression[a]	
		Northern analysis	PCR analysis
Nude mouse tumor cell lines	AF6295[b]	++++	++++
	AF3642[b]	++++	++++
Hematopoietic cell lines			
Lymphoid	LAM	ND	−
	DHL-4	−	−
Myeloid			
Promyelocytic	HL60	−	−[c]
Acute myelogenous leukemia	SKL1	−	++
CML blast phase	KOPM-28	−	++
	EM2	−	+
	EM3	−	−
	K562	−	++
Primary hematopoietic tissues	NPB 1[d]	ND	−
	NPB 2	−	+
	CML/CP[e]	−	+
	MDS 1[f]	−	−
	MDS 2	−	+
Breast cell lines	MDA-157	−	−
	MDA-468	−	+++
	SK-BR3	−	−
	BT-20	−	++
	BT-474	−	+
	MCF-7	−	+
	600 PEI	−	++
Normal breast epithelia	337	−	++
Miscellaneous			
Cervical cancer	HeLa	+++	+++
Lung cancer	A549	+++	+++
Epidermoid cancer	A431	+++	+++
Normal human fibroblast cell lines	WI 38	−	+++
	BG-9	+++	+++
	IMR-90	+++	+++
Mouse fibroblast cell line	NIH 3T3	+	+++[g]

[a] Semiquantitated as follows: ++++, very high; +++, high; ++, moderate; +, weak; −, undetectable. ND, not determined.
[b] Derived from secondary nude mouse tumors arising from transfection of DNA from a patient with blast crisis CML (AF6295) or a patient with chronic-phase CML (AF3642).
[c] The fragment detected was approximately 600 bp. The sequence of this fragment was distinct from that of the *axl* protein and may represent a related kinase.
[d] RNA was extracted from normal peripheral blood leukocytes.
[e] RNA was extracted from peripheral leukocytes from a patient with chronic-phase CML.
[f] RNA was extracted from peripheral leukocytes from a patient with myelodysplasia.
[g] Analysis was done with use of only *axl* primers specific for the kinase domain.

with other known kinases reveals interesting structural similarities. As with the insulin receptor family, the *eph*, *eck*, and *elk* kinases contain a cysteine-rich region juxtaposed to FNIII repeats (Fig. 4). However, in contrast to the insulin receptor family, the *eph*, *eck*, and *elk* kinases are characterized by a vestigial IgL repeat near their amino termini and do not contain proteolytic processing sites for the formation of α and β subunits (Fig. 3 and 4). Thus the *eph* family of kinases represents a related but distinct subclass within the insulin receptor family. By amino acid homology, the *axl* protein is most similar to the insulin receptor family. However, the extracellular domain of the *axl* protein lacks a cysteine-rich region and possesses the unique juxtaposition of two IgL and two FNIII repeats, indicating that this protein is a separate subclass within the insulin receptor kinase family. Although not previously described, we have observed that the extracellular domain of the *trk* protein also contains IgL and FNIII repeats, making it similar in structure to the *axl* protein. Interestingly, the FNIII repeats in the *trk* protein closely resemble IgL repeats (Fig. 3). Bazan has observed that IgL and FNIII domains are similar in overall structure, suggesting that these domains evolved from a common ancestral gene (5). It is possible, therefore, that the *trk* protein represents an ancestral kinase with domains intermediate between IgL and FNIII repeats. In view of these sequence associations, we propose that the *eph*, *eck*, and *elk* proteins represent one subclass and the *axl* and *trk* proteins represent another subclass of receptor tyrosine kinases that are evolutionarily related to the insulin receptor family (Fig. 4).

In contrast to other oncogenic receptor kinase genes such as *ret*, *trk*, and *fms* (CSF-1R), the normal c-*axl* gene was activated by overexpression rather than by genomic alterations such as mutation or rearrangement. Overexpression of normal receptors (e.g., *erbB2*) can also result in neoplastic transformation in an apparently ligand-independent fashion (16). Transfection of *axl* cDNA into 3T3 cells indicates that selection for cells overexpressing *axl* is required for manifestation of the transformed phenotype; low-level expression is insufficient for neoplastic transformation. Furthermore, we have identified two distinct variants of *axl* receptor present in both the nude mouse tumor cell lines and normal cells but expressed at different ratios in these samples. Presumably these two forms are derived by differential splicing of an alternative exon. Similar structural variation is

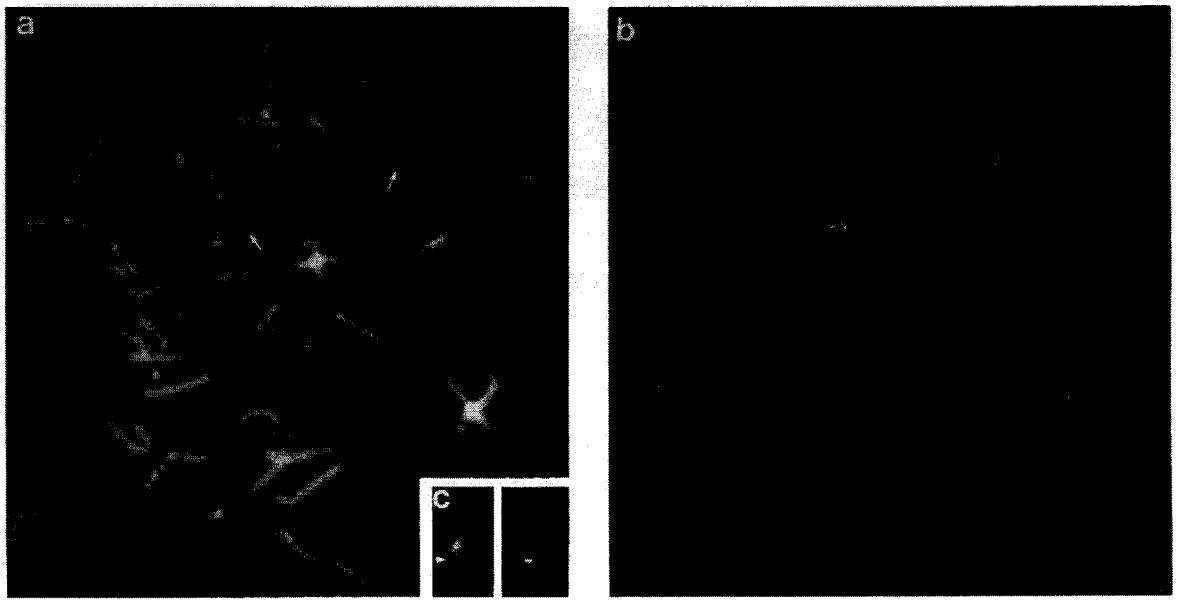

FIG. 10. In situ hybridization of a biotin-labelled *axl* probe to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. (A) Counterstained with 2,4-diamidino-2-phenylindole dihydrochloride; (B) detection of the probe with fluorescein isothiocyanate-conjugated avidin (45). The chromosome 19 homologs are identified with arrows; specific labelling was observed at 19q13.2. (C) Partial karyotype of a chromosome 19 homolog illustrating specific labelling at 19q13.2.

seen in the generation of the insulin receptor in which a 36-nucleotide exon (exon 11) coding for a portion of the extracellular domain is spliced into the transcript in some tissues and not in others (67). Though the function of this differential splicing event in both the insulin receptor and the *axl* protein remains unclear, our data indicate that both forms of the *axl* protein are biologically indistinguishable in terms of their transforming capacity in NIH 3T3 cells. This also suggests that the substitution of leucine for glutamic acid at amino acid 338 represents a sequence polymorphism as opposed to an activating mutation. This view is further supported by the finding that another cDNA clone (clone 4-9) from the normal human fibroblast cDNA library has the same sequence as clone 1-4 with regard to amino acid 338 and the presence of the 27-base insert. These observations are consistent with the conclusion that *axl* transformation is due to overexpression of a normal receptor tyrosine kinase. Similar to *axl*, the *eph* gene has been shown to have weak transforming properties when overexpressed in 3T3 cells (51).

Several lines of evidence underscore the potential role of *axl* in normal cellular function. First, the transforming activity of *axl* suggests a role for the proto-oncogene in proliferation. Second, *axl* expression can be detected in the majority of cell types examined, implying a significant role for *axl* in normal cellular activity. This notion is further supported by the near-ubiquitous expression of *axl* message in various mouse organs (58a). Third, *axl* expression is altered in hematopoietic differentiation. *axl* RNA levels are augmented >10-fold in the CML cell line K562 upon addition of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate), which induces megakaryoblastic differentiation in these cells (57a, 74). Using a PCR-based cloning strategy, Partanen et al. (60) have detected novel tyrosine kinases expressed in differentiated K562 cells. One of these partial cDNA clones, designated JTK 11, has the same sequence as the *axl* protein at amino acids 669 through 736 in the kinase domain (60).

The specific role of *axl* in the pathogenesis of CML remains unclear. However, our data suggest that *axl* is a CML-associated oncogene. First, identical forms of *axl* emerged as the transforming gene in two of eight non-mutant *ras*-bearing CML cases analyzed in a transfection-tumorigenicity assay (47). We have applied this assay on >40 DNAs from numerous malignancies (breast carcinomas, squamous cell carcinomas of the skin, renal cell carcinomas, and other types of leukemias) and have not uncovered *axl* in any transformant (46a). Thus, an oncogenic *axl* gene has been found only in CML samples. Since *axl*-induced transformation of 3T3 cells results from overexpression of the transfected proto-oncogene, these two CML samples may contain mutations in the *axl* promoter or as yet uncloned 5′ untranslated sequences which may result in overexpression of the *axl* kinase. Activation of the *lck* proto-oncogene occurs through a similar mechanism in which mutations in the 5′ untranslated region increase the translational efficiency of the gene, resulting in overexpression of the oncoprotein (48). Characterization of the *axl* promoter region will address this possibility. In addition, using chromosomal in situ hybridization (45), we have localized *axl* to chromosome 19, band q13.2 (Fig. 10), where another oncogene, *bcl3*, is also found (19q13.1-13.2) (42, 54). Interestingly, chromosome 19 abnormalities (especially trisomy 19) are frequently associated with the conversion of chronic-phase CML to blast phase occurring in approximately 18% of blast crisis samples (64). Future studies will help define *axl*'s role in human malignancies.

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ADDENDUM

Using the same transfection assay as described in this report, Janssen et al. (35a) have identified the identical gene, which they call UFO, in DNA from a patient with chronic myeloproliferative disease.

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